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珍稀濒危植物硬叶兜兰的遗传多样性及遗传结构研究*

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摘要: 由于人为采集、走私贩卖以及生境的破坏, 分布于中国西南石灰岩地区的野生硬叶兜兰居群受到严重的干扰与威胁。为有效地保护这种珍稀野生植物, 本研究采用 ISSR 和 SRAP 两种分子标记对 15 个硬叶兜兰野生居群进行遗传多样性及遗传结构的研究。结果表明, 硬叶兜兰在物种水平上具有较高的遗传多样性 (ISSR: PPB = 91.66%, H_e = 0.3839; SRAP: PPB = 99.29%, H_e = 0.2806)。硬叶兜兰居群间存在一定程度的遗传分化 (ISSR: G_{st} = 0.2577; SRAP: G_{st} = 0.2383), 可能由于较低的基因流 (ISSR: Nm = 0.7201; SRAP: Nm = 0.7991) 所致。UPGMA 聚类分析以及主成分分析均把 15 个居群分成 2 个主要分支。居群间的地理距离和海拔差距是引起居群遗传分化的自然因素。

关键词: 资源保护; 分子标记; 遗传多样性; 遗传分化; 硬叶兜兰
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ISSR and SRAP Markers Reveal Genetic Diversity and Population Structure of an Endangered Slipper Orchid, *Paphiopedilum micranthum* (Orchidaceae)

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Abstract: *Paphiopedilum micranthum* is an endangered pink slipper orchid mainly distributed in the limestone areas of southwestern China. Wild populations of this species have been seriously threatened by excessive collections, rampant smuggling for export, and habitat destruction. We used 15 ISSR markers and 11 SRAP markers to investigate the genetic diversity and structure of 15 natural populations. A high degree of diversity was observed at the species level (ISSR: PPB = 91.66%, H_e = 0.3839; SRAP: PPB = 99.29%, H_e = 0.2806). Certain degree of genetic differentiation among populations (ISSR: G_{st} = 0.2577; SRAP: G_{st} = 0.2383) was detected maybe caused by low gene flow (ISSR: Nm = 0.7201; SRAP: Nm = 0.7991). Consistent with the results of Principal Coordinate Analysis, the UPGMA dendrogram analysis divided the 15 populations into two main clades. In addition to geographic distance, the difference in elevation was another natural factor contributing to this differentiation. Knowledge about genetic diversity and structure gained from our study will be beneficial for the development of reasonable and efficient strategies to conserve this endangered species.

Key words: Conservation; DNA markers; Genetic diversity; Genetic differentiation; *Paphiopedilum micranthum*

The genus *Paphiopedilum* Pfitz., a primitive group within Orchidaceae, is distributed from southern China

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to New Guinea in tropical and subtropical Asia (Liu *et al.*, 2009). These slipper orchids are named for the shape of the deeply saccate lip of their flowers. Growers prize them for their very great ornamental value, particularly because these small plants have spectacular, large flowers (Cribb, 1998). More than 20 000 slipper orchid hybrid grexes have been registered, demonstrating their remarkable rise in popularity (Zeng *et al.*, 2010). Provinces in southwestern China, including southeastern Yunnan, northern and western Guangxi, and southwestern Guizhou, are known as diversity and evolutionary hotspots for this genus (Luo *et al.*, 2003; Liu *et al.*, 2010). There are 25 of 80 species grow on limestone hills in that region. In addition to threats from rapid habitat destruction associated with faster economic growth and rural development during the past three decades, wild slipper orchids are facing destructive collecting pressures due to large horticultural and commercial demands in China (Luo *et al.*, 2003; Liu *et al.*, 2009). This vulnerability by *Paphiopedilum* species to environmental change can be also traced to the limited distribution of many species in the wild and their occurrence in populations that often contain only a few plants each (Cribb and McGough, 1997). It is urgent to take proper strategies to conserve these endangered species.

Paphiopedilum micranthum is restricted to the limestone hills of southwestern China. In the early 1980s, plants were exported from China and deservedly received First Class Certificates from the American Orchid Society, the Royal Horticultural Society, and many other awarding groups because these beautiful plants have pink flowers that are larger than those previously known (Cribb, 1998). Since then, this species has been cultivated in large quantities while wild populations have declined sharply (Li *et al.*, 2002b). To preserve and exploit *P. micranthum* and its allied species, multiple studies have been carried out including the effects of conservation efforts (Liu *et al.*, 2004; 2006), physiological ecology (Chang *et al.*, 2011), pollination biology (Shi *et al.*, 2007), and breeding strategies (Chen *et al.*, 2004; Liao *et al.*, 2011; Chung and Choi, 2012).

Clarifying genetic diversity and population structure of extant populations not only provides insights into the evolutionary and demographic history of threatened species (Hamrick *et al.*, 1982; Hamrick and Godt, 1990), but also facilitates the design of effective conservation and management strategies (Jian *et al.*, 2006). However, only a few studies have been conducted because of the difficulty associated with collecting population samples for slipper orchids, including one that used random amplified polymorphic DNA (RAPD) markers to examine the genetic diversity within four naturally distributed *P. micranthum* populations (Li *et al.*, 2002b).

Inter-simple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) are relatively simple and highly reproducible marker technologies that do not rely on prior information about a DNA sequence, and which require very little starting DNA template (Zietkiewicz *et al.*, 1994; Li and Quiros, 2001). These two systems have proven to be powerful and efficient techniques for analyses of population genetics diversity, molecular taxonomic classification, and marker-assisted breeding in many orchid species (Smith *et al.*, 2002; Wallace, 2003; Ding *et al.*, 2008; George *et al.*, 2009; Wang *et al.*, 2009; Cai *et al.*, 2011). We thus apply these two markers to investigate the genetic diversity and population structure of *P. micranthum* in southwestern China. Our main goals were to 1) determine the level of genetic variation within and among populations, 2) characterize the extent of genetic differentiation between populations, and 3) identify the causes for this observed differentiation.

1 Materials and methods

1.1 Sampling and plant material collection

We collected 407 individuals of *Paphiopedilum micranthum* from 15 wild populations in three provinces of southwestern China (Fig. 1; Table 1). Because this species is rhizomatous and forms clumps

of multiple plants in the wild (Tsi *et al.*, 1999), we tried to avoid collecting ramets of only a single genotype by randomly selecting individuals that were spaced at least 3 m apart. Young leaves were harvested and dried by silica gel for further DNA isolation. The genomic DNA of each sample was extracted from leaves by the standard CTAB (cetyltrimethyl ammonium bromide) method of Doyle and Doyle (1987).

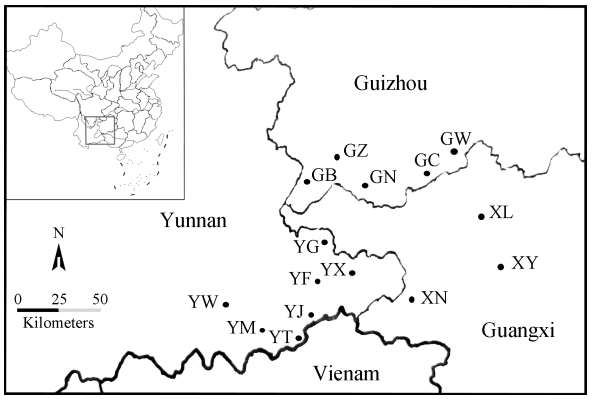

Fig. 1　Sampling sites for wild populations of *Paphiopedilum micranthum* in southwestern China. Population codes are explained with Table 1

1.2　ISSR fingerprinting

The sequences for our ISSR primers were provided by the Biotechnology Laboratory, University of British Columbia, Canada. There were 15 (Table 2) from 90 arbitrary primers showing good repetition, special bands, and distinct polymorphism. Each ISSR reaction mixture (20 μL) contained 50 ng of template DNA, 8 μL of 2×Taq PCR Master Mix (0.1 U of Taq polymerase per μL, 0.5 mmol·L^{-1} dNTP, 20 mmol·L^{-1} Tris-HCl, 100 mmol·L^{-1} KCl, and 3 mmol·L^{-1} $MgCl_2$), plus 2% formamide, 100 nmol·L^{-1} primer, and double-distilled water. The PCR program included an initial denaturation at 94 ℃ for 5 min; then 40 cycles of 94 ℃ for 45 s, 53 ℃ for 1 min, and 72 ℃ for 2 min; followed by a final extension at 72 ℃ for 7 min. The ISSR-PCR products were separated in 2.0% agarose gels buffered with 0.5×TBE. A 100 bp DNA ladder (Fermentas) was used as a size marker. After staining with ethidium bromide, the DNA fragments were identified by image analysis software for gel documentation (Lab Works Software, version 3.0; UVP, Upland, CA, USA).

1.3　SRAP fingerprinting

The SRAP marker analysis was performed as described by Li and Quiros (2001). From 88 arbitrary primer pairs, we selected 11 combinations that showed distinct polymorphism (Table 2). Each SRAP reaction mixture (20 μL) contained 40 ng of template DNA, 8 μL of 2×Taq PCR Master Mix, 100 nmol·L^{-1} each for forward and reverse primers, and double-distilled water. Amplification was performed under the following conditions: 94 ℃/3 min; five cycles of 94 ℃/1 min, 35 ℃/1 min, 72 ℃/1 min; then 30 cycles

Table 1　Sampling information for 15 *Paphiopedilum micranthum* populations

Population code	Location	Number of samples	Elevation/m	Longitude (E)	Latitude (N)
YW	Wenshan, Yunnan	36	1550	104°18′42″	23°10′22″
YG	Guangnan, Yunnan	36	1530	105°04′15″	24°11′31″
YT	Tianbao, Yunnan	24	1350	104°44′26″	23°03′41″
YX	Xichou, Yunnan	27	1540	104°31′51″	23°20′13″
YF	Fadou, Yunnan	29	1580	104°45′19″	23°24′11″
YJ	Jinchang, Yunnan	24	1490	104°49′17″	23°07′47″
YM	Maguan, Yunnan	21	1438	104°22′20″	22°56′48″
XL	Leye, Guangxi	23	1020	106°21′14″	24°48′54″
XN	Napo, Guangxi	24	1077	105°57′21″	23°24′29″
XY	Linyun, Guangxi	19	1050	106°44′13″	24°34′14″
GC	Ceheng, Guizhou	29	1273	105°39′59″	24°59′22″
GN	Nidang, Guizhou	29	1479	104°48′24″	24°49′49″
GW	Wangmo, Guizhou	28	1274	106°23′12″	25°14′54″
GZ	Zhaojiadui, Guizhou	30	1375	105°00′23″	25°01′57″
GB	Bajie, Guizhou	28	1182	104°59′41″	24°54′39″

Table 2 Sequences of 15 ISSR primers and 11 SRAP Primers

ISSR primers	Sequences	SRAP primer combinations	Forward primer	Reverse primer
UBC811	$(GA)_8C$	Me1-Em9	TGAGTCCAAACCGGATA	GACTGCGTACGAATTCGA
UBC814	$(CT)_8A$	Me2-Em3	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTGAC
UBC815	$(GA)_8G$	Me3-Em11	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTCCA
UBC822	$(TC)_8A$	Me5-Em2	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTTGC
UBC823	$(TC)_8C$	Me5-Em5	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTAAC
UBC825	$(AC)_8T$	Me5-Em7	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTCAA
UBC827	$(TC)_8G$	Me5-Em9	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTCGA
UBC834	$(AG)_8YT$	Me6-Em3	TGAGTCCAAACCGGTAA	GACTGCGTACGAATTGAC
UBC835	$(AG)_8YC$	Me6-Em4	TGAGTCCAAACCGGTAA	GACTGCGTACGAATTTGA
UBC844	$(CT)_8RC$	Me7-Em10	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTCAG
UBC845	$(CT)_8RG$	Me8-Em3	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTGAC
UBC853	$(TC)_8RT$			
UBC857	$(AC)_8YG$			
UBC859	$(CG)_8RC$			
UBC873	$(GACA)_4$			

of 94 ℃/1 min, 50 ℃/1 min, 72 ℃/1 min; and a final extension of 72 ℃/10 min. All PCR reactions were run in the ABI 2720 Thermal Cycler. The SRAP-PCR products were analyzed on 6% non-denatured polyacrylamide gels in 1 × TBE buffer running at 380 V constant voltage for 1.0 h. Afterward, silver-staining was done as reported by Bassam *et al.* (1991).

To ensure the reliability of the genotype, negative controls were run at each step to check for exogenous contamination for ISSR and SRAP. The experiment was repeated twice, and only data from intensely stained, unambiguous bands were used for statistical analysis.

1.4 Data analysis

Amplified bands were scored according to the presence (1) or absence (0) of homologous bands for all samples, and were displayed as part of a binary matrix. These data were analyzed by POPGENE version 1.32 (Francis and Yang, 2000) to estimate the degree of genetic diversity in *P. micranthum*. Some essential diversity parameters, e.g., percentage of polymorphic bands (PPB), Shannon's information index I (Shannon and Weaver, 1949), and Nei's genetic diversity H_e (Nei, 1978), were evaluated at both the population and species levels. Genetic differentiation between populations was estimated by the coefficients for genetic differentiation (G_{st}) and gene flow (N_m) (Slatkin and Barton, 1989). To examine the genetic relationship among populations, we generated a genetic distance map via POPGENE. We also constructed a dendrogram per Nei's (1978) genetic distance method, using the unweighted pair-group method of averages (UPGMA) and 1 000 permutations of bootstrapping, with TFPGA version 1.3 (Miller, 1997). A Mantel test was performed to estimate any correlations between the matrices of genetic distances and geographical distances, using GenAlEx version 6.5 (Peakall and Smouse, 2006). Principal Coordinate Analysis (PCoA) was also conducted with GenAlEx version 6.5, based on the calculated Jaccard's similarity coefficients. Correlations between elevational differences and genetic distances were estimated by PASSAGE version 2 (Rosenberg and Anderson, 2011).

2 Results

2.1 Genetic diversity in populations

A total of 88 different bands were scored from 15 ISSR primers, of which 81 were polymorphic (92.04%). Our 11 SRAP primer combinations produced 280 bands, from 100 to 500 bp long, across all 407 individuals. Of those, 278 were polymorphic (99.29%). A summary of the ISSR and SRAP data from each *Paphiopedilum micranthum* population is

presented in Table 3. The ISSR analyses resulted in a H_e value of 0.3839 and an I value of 0.5646 at the species level. Within each population, the PPB varied from 64.89% (YJ and XL) to 89.36% (YW). The mean H_e was 0.2847, ranging from 0.1975 (XL) to 0.3238 (YW). Values for I showed similar trends, ranging from 0.3024 (XL) to 0.4799 (YW). The SRAP analyses produced a H_e value of 0.2806 and an I of 0.4359 at the species level. Within each population, the PPB varied from 62.54% (XL) to 82.69% (YW). Values for H_e were 0.1963 to 0.2410 (mean 0.2166); for I, 0.2974 to 0.3701 (mean 0.3301). These data from both ISSR and SRAP showed that, among the 15 populations, the genetic diversity of *P. micranthum* was richest within YW and poorest within XL.

2.2 Genetic differentiation within and among populations

AMOVA analysis with GenAlEx 6.5 software presented significant genetic variation ($P<0.001$) among and within the 15 populations. Based on ISSR analysis, the main component (69%) within the total molecular variance was attributed to differences between individuals within populations, with the remainder (31%) coming from among populations (Table 4). This was consistent with the POPGENE results ($G_{st}=0.2577$). Similarly, when the SRAP data were analyzed, moderate genetic differentiation ($G_{st}=0.2383$) was found among populations. The average number of individuals exchanged between populations per generation (Nm) was 0.7201 based on ISSR markers and 0.7991 when SRAP markers were used (Table 3). This indicated that limited pollen and seed dispersal occurred among populations.

2.3 Genetic distances among populations

The Mantel tests showed significant positive correlations between genetic and geographic distances for both ISSRs ($r=0.455$; $P=0.001$) and SRAPs

Table 3　Genetic diversity of *Paphiopedilum micranthum* based on ISSR and SRAP analyses

Population	ISSR					SRAP				
	PPB/%	H_e	I	G_{st}	Nm	PPB/%	H_e	I	G_{st}	Nm
YW	89.36	0.3238	0.4799			82.69	0.2410	0.3701		
YG	87.23	0.3087	0.4598			68.90	0.2015	0.3076		
YT	77.66	0.2873	0.4239			64.66	0.2084	0.3159		
YX	80.85	0.2831	0.4196			75.27	0.2177	0.3346		
YF	75.53	0.2787	0.4113			70.67	0.2154	0.3274		
YJ	64.89	0.2314	0.3448			63.60	0.2053	0.3106		
YM	87.23	0.3220	0.4767			67.14	0.2040	0.3095		
XL	64.89	0.1975	0.3024			62.54	0.1963	0.2974		
XN	75.51	0.2602	0.3907			64.32	0.2199	0.3289		
XY	77.66	0.2851	0.4219			69.26	0.2136	0.3301		
GC	81.91	0.3026	0.4468			71.38	0.2227	0.3384		
GN	87.23	0.3177	0.4694			74.91	0.2273	0.3479		
GW	82.98	0.2838	0.4255			79.86	0.2362	0.3650		
GZ	88.30	0.3035	0.4506			79.95	0.2384	0.3651		
GB	82.98	0.2912	0.4312			67.49	0.2018	0.3077		
Mean	80.28	0.2847	0.4236			70.67	0.2166	0.3301		
Species level	91.66	0.3839	0.5646	0.2577	0.7201	99.29	0.2806	0.4359	0.2383	0.7991

Table 4　AMOVA results for genetic variance within and among populations by ISSR and SRAP

Markers	Source of variation	df	Sum of squares	Mean squares	Variation components	Total variation/%	P-value
ISSR	Among populations	14	2425.072	173.219	5.918	31	<0.001
	Within populations	392	5086.606	12.976	12.976	69	<0.001
SRAP	Among populations	14	4569.319	326.380	10.857	25	<0.001
	Within populations	392	12709.875	32.423	32.423	75	<0.001

($r=0.421$; $P=0.003$) (Table 5). To examine further why genetic distances formed among these populations, we used a Mantel test with PASSAGE to investigate any correlation between elevational differences and genetic distances. Here, positive correlations between those two components were identified from both ISSR analysis ($r=0.4356$; $P=0.0006$) and SRAP analysis ($r=0.3953$; $P=0.0025$).

The UPGMA analyses applying both ISSRs (Fig. 2A) and SRAPs (Fig. 2B) clearly resolved the 15 populations into two major clusters. Cluster I included 12 populations from Guizhou, Guangxi, and Yunnan provinces while Cluster II comprised the XL, GW, and XY populations from Guizhou and Guangxi provinces. The PCoA results of ISSR and SRAP data were shown in Figure 3A and Figure 3B, respectively. Consistent with the UPGMA dendrogram, PCoA also revealed that the 15 populations could be separated into two main clusters.

Table 5 Mantel tests to evaluate genetic distances, geographic distances, and elevational differences for 15 *Paphiopedilum micranthum* populations

	Genetic distance versus Geographic distance		Genetic distance versus Elevational difference	
	P	*r*	*P*	*r*
ISSR	0.455	0.001	0.436	0.001
SRAP	0.421	0.003	0.395	0.003

3 Discussion

3.1 Genetic diversity

We collected 407 samples of *P. micranthum* from native 15 populations distributed in southwestern China and analyzed their genetic variation in this study. The ISSR and SRAP markers revealed higher genetic diversity than those of other orchids, e. g., *Cymbidium goeringii*, *Goodyera procera*, *Changnienia amoena*, and *Cypripedium flavum* (Table 6). Moreover,

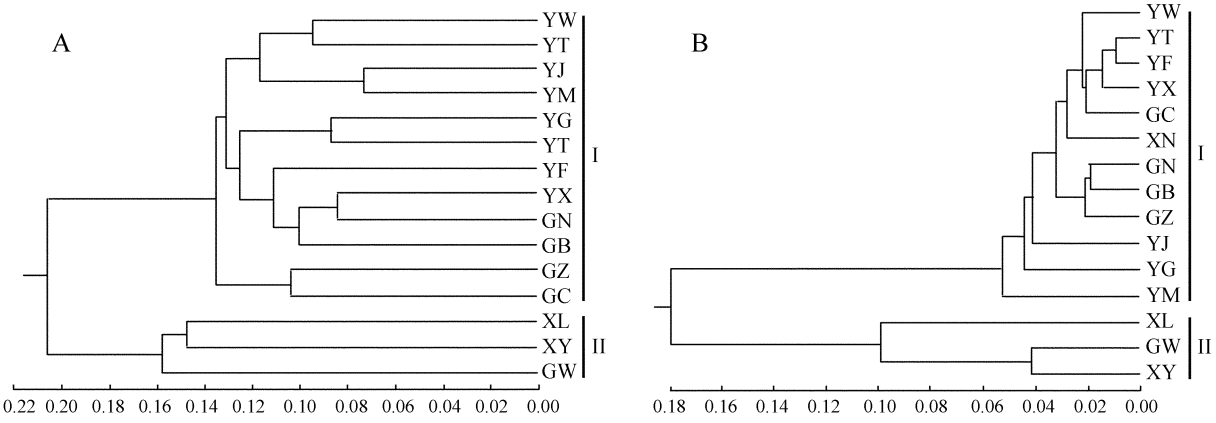

Fig. 2 A. UPGMA dendrogram based on ISSR data; B. UPGMA dendrogram based on SRAP data

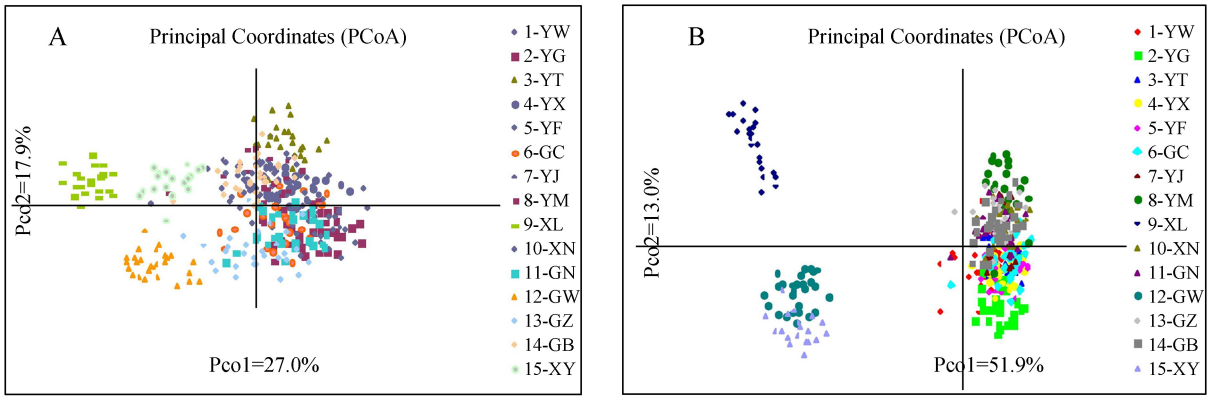

Fig. 3 A. Principal Coordinate Analysis of 407 individuals, based on ISSR data; B. Principal Coordinate Analysis of 407 individuals, based on SRAP data

Table 6　Comparisons of values for genetic diversity (PPB and H_e) and differentiation (G_{st}) between *Paphiopedilum micranthum* and other orchid species

Species	PPB/%	H_e	G_{st}	Markers	References
Paphiopedilum micranthum	91.66	0.3839	0.2577	ISSR	This paper
	99.29	0.2806	0.2383	SRAP	This paper
P. micranthum	73.30	0.2170	——	RAPD	Li *et al.*, 2002b
Cypripedium flvum	82.69	0.2884	0.1540	AFLP	Cai *et al.*, 2008
Cypripedium calceolus	36.40	0.1490	0.0590	Allozyme	Brzosko *et al.*, 2011
Cymbidium goerigii	88.19	0.2628	0.2440	ISSR	Gao and Yang, 2006
Goodyera procera	97.03	0.2930	0.3900	RAPD	Wong and Sun, 1999
Changnienia amoena	76.50	0.1941	——	RAPD	Li *et al.*, 2002a
Orchidaceae (average)	——	——	0.1870	——	Forrest *et al.*, 2004

the degree of diversity for *P. micranthum* found here was greater than that calculated when RAPD markers were used with four previous populations of that species (PPB=73.3%; H_e=0.217) (Li *et al.*, 2002b). The primary reason for this discrepancy may lie in the choice of markers. The polymorphism of products was higher with ISSRs and SRAPs than with RAPDs, thereby providing more information about a particular genome. Moreover, ISSR and SRAP molecular markers are considered more stable and reliable when compared with the RAPD technique (Dirlewanger *et al.*, 1998; Esselman *et al.*, 1999; Gilbert *et al.*, 1999). Another explanation is that we used more populations and individual samples than were involved in an earlier investigation by Li *et al.* (2002b). Some genetic parameters (H_e) are sensitive to sample size, thus influencing how well those parameters can be estimated (Li *et al.*, 2000).

Plants of *P. micranthum* are perennial and herbaceous. Their decades-long life spans may contribute to their high genetic diversity (Nybom, 2004). Moreover, the breeding mode is an important factor affecting genetic diversity (Hamrick, 1982; Hamrick and Godt, 1990). This species reproduces by both sexually and asexually, which has been considered as a strategy to maximize heterozygosity and reproductive success (Yan *et al.*, 1999). One capsule of *P. micranthum* contains more than 5 000 seeds, which can ensure a large gene pool that can provide abundant heterozygotes (Luo *et al.*, 2003). In addition, *P. micranthum* has strong capacity of clonal reproduction, developing rhizomes that often form numerous ramets and support an apparent plexiform distribution (Tsi *et al.*, 1999). Although a pollination system has not been reported for *P. micranthum*, Cribb and McGough (1997) has classified it as essentially an outbreeding species that utilizes insect pollination. Then, its out-crossing strategy might also contribute to its higher diversity comparing with that of inbreeding orchid species (Ehlers and Pedersen, 2000).

3.2　Population genetic structure

Based on the assumption of Hardy-Weinberg equilibrium, we detected moderate genetic differentiation among our populations of *P. micranthum* (G_{st}, 0.2577 by ISSR; 0.2383, by SRAP). AMOVA analysis also showed that 31% (ISSR) and 25% (SRAP) genetic variation existed among populations, as well as 69% (ISSR) and 75% (SRAP) variation within populations (Table 4). This differentiation is higher comparing with affinitive, insect-pollinated orchid species, such as *Cypripedium calceolus* (G_{st}=0.059; Brzosko *et al.*, 2011) and *C. flavum* (G_{st}=0.154; Cai *et al.*, 2008), for which seeds are wind-dispersed. Forrest *et al.* (2004) have reported that orchid G_{st} values range from 0.012 to 0.924 (average 0.187), and that variations in population genetics differentiation are huge between orchid species. As shown here, the G_{st} values for *P. micranthum* (0.2577 and 0.2383) were higher than the average of 0.187 calculated for other orchid species.

The genetic structure of a plant species is usually influenced by factors such as mating system and extent of gene flow (Hamrick and Godt, 1990). The theory of population genetics suggests that low *Nm*

values (i. e., <1) cannot prevent the differentiation between populations that is caused by genetic drift (Wright, 1931; Hartl and Clark, 1989). Our study produced *Nm* values of 0. 7201 (ISSR) and 0. 7991 (SRAP), both less than 1, which aggravated the differentiation between populations. The distribution of pollen and seeds is a major determinant of gene flow in natural populations (Li and Chen, 2004). Because most orchid species rely primarily on wind dispersal, their seeds can move across great distances (Swamy *et al.*, 2004, 2007). In contrast to related species of *Paphiopedilum* and *Cypripedium*, however, seeds of *P. micranthum* are not carried as far (Zhang, 2012). This might explain why genetic differentiation between populations is relatively high in that species. Another factor in the reduction of gene flow between populations is anthropogenic. Because of their high ornamental value, most of the older, flowering plants have been collected from wild populations, leaving behind only the younger specimens (Luo *et al.*, 2003).

3.3 Genetic relationships among populations

Both the UPGMA and PCoA evaluations divided the 15 geographical populations of *P. micranthum* into two clusters, with one comprising 12 populations in Yunnan and Guizhou and the other containing two populations in Guanxi plus one in Guizhou. PCoA revealed high genetic similarity in Cluster I, whereas Cluster II showed evidence of genetic isolation among its three populations. Results of Mantel tests based on data from ISSR (r = 0. 455, P = 0. 001) and SRAP (r = 0. 421, P = 0. 003) indicated that genetic distance was correlated with geographic distance, a phenomenon that is commonly found in endemic and endangered species (Godt *et al.*, 2005, Luan *et al.*, 2006). Although *P. micranthum* is not itself endemic to China, it is considered endangered and protected there.

Genetic differentiation between natural populations is usually related to geographical barriers, such as high mountains and rivers, which make gene exchange almost impossible to achieve from one population to another (Godt *et al.*, 2005). Here, however, we could not identify any such barrier, other than elevational difference, between Clusters I and II. Our Mantel tests detected a remarkable correlation (P<0. 05) between genetic distance and elevation. Many studies have shown that elevation-associated temperature variations play an important role in directing genetic diversity and differentiation (Bayer, 1992; Li and Chen, 2004; Jiang *et al.*, 2005). For example, the genetic variation among *Rhodiola angusta* populations at Changbai Mountain increases as the temperature decreases due to elevation (Yan *et al.*, 1999). Likewise, apart from geographical distance, differences in elevation may be an important factor that divided our *P. micranthum* populations into two genetic clusters.

3.4 Conservation recommendations

For endangered species, the goals of conservation are to ensure the continued survival of populations and to maintain their evolutionary potential (Hamrick and Godt 1990; Wong and Sun 1999). Given our findings, individuals of Cluster II comprised the XL, GW, and XY populations from Guizhou and Guangxi provinces have relatively low genetic variation. Therefore, we recommend that these populations be prioritized for conservation protection. Meanwhile, it is necessary to carry out the *ex*-situ conservation and the artificial reproduction of *P. micranthum* as soon as possible, to store up mass seedlings of artificial reproduction prepared for wild re-introduction, to renew this endangered species and to make it thriving.

4 Conclusion

Using ISSR and SRAP molecular markers, we determined that plants of *P. micranthum* exhibit a high degree of genetic diversity, and that genetic differentiation is moderate among natural populations. When our sample populations were assigned to two clusters, the one representing sites in Yunnan and Guizhou showed high diversity while that of the Guangxi sites had low diversity. The genetic differentiation

between populations was related to variations in biological characteristics, such as capacity for seed dispersal, as well as associations with geographical distance and elevational differences. Our results will be beneficial to managers who can develop reasonable strategies for conserving this endangered slipper orchid.

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